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The *Arabidopsis* TOR kinase links plant growth, yield, stress resistance and mRNA translation

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Plants, unlike animals, have plastic organ growth that is largely dependent on environmental information. However, so far, little is known about how this information is perceived and transduced into coherent growth and developmental decisions. Here, we report that the growth of *Arabidopsis* is positively correlated with the level of expression of the TARGET OF RAPAMYCIN (TOR) kinase. Diminished or augmented expression of the *AtTOR* gene results in a dose-dependent decrease or increase, respectively, in organ and cell size, seed production and resistance to osmotic stress. Strong downregulation of *AtTOR* expression by inducible RNA interference also leads to a post-germinative halt in growth and development, which phenocopies the action of the plant hormone abscisic acid, to an early senescence and to a reduction in the amount of translated messenger RNA. Thus, we propose that the AtTOR kinase is one of the contributors to the link between environmental cues and growth processes in plants.

Keywords: target of rapamycin; *Arabidopsis*; organ growth; mRNA translation; abscisic acid

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INTRODUCTION

The modulation of growth rate in response to environmental cues such as nutrient availability is essential for survival. The main function of the conserved eukaryotic target of rapamycin (TOR) kinase is to promote cell growth in response to favourable conditions. In yeast and animal cells, TOR regulates numerous biological processes, including transcription and translation of ribosomal components, which collectively contributes to cell growth. The TOR kinase exists in at least two large protein complexes that act in recruiting its various substrates (Martin & Hall, 2005; Wullschleger *et al*, 2006). The first complex includes the TOR (TOR1 or TOR2 in yeast), RAPTOR/KOG1 and mLST8/GβL proteins, and is sensitive to rapamycin; the second complex contains TOR (TOR2 in yeast), Rictor/AVO3 and mLST8/GβL, and phosphorylates Akt/PKB in animal cells.

As immobile organisms, plants need to constantly adjust their growth—defined as an increase in organ size—and development—production of new organs—to nutritional information such as light, water or ion availability; however, the genes and mechanisms involved in the perception and transduction of these information are so far poorly known. Several components of the TOR signalling pathway are present in *Arabidopsis*, and are ideal candidates for operating the link between nutritional sensing and the regulation of growth (Menand *et al*, 2004). Some members of the TOR complexes such as the RAPTOR (Anderson *et al*, 2005; Deprost *et al*, 2005) and mLST8/GβL proteins exist in plants. There is also evidence that possible TOR substrates and regulators, such as S6 or other AGC kinases (Turck *et al*, 2004) and the 3-PHOSPHOINOSITIDE-DEPENDENT PROTEIN KINASE 1 (PDK1), are conserved in plants (Deak *et al*, 1999; Bögre *et al*, 2003; Otterhag *et al*, 2006). Furthermore, it has been reported that *Arabidopsis* PDK1 phosphorylates S6 kinase 1 and that, as in animal cells, RAPTOR interacts *in vivo* both with S6 kinase and with TOR through the HEAT repeats (Otterhag *et al*, 2006; Mahfouz *et al*, 2006).

Unlike algae such as *Chlamydomonas* (Crespo *et al*, 2005), higher plants seem to be resistant to rapamycin (Menand *et al*, 2002); therefore, the use of rapamycin to inhibit the activity of plant TOR has been precluded. Fortunately, several large collections of insertion mutants are available in *Arabidopsis*, and we have previously shown that disruption of the unique *AtTor* gene causes, as in *Drosophila* (Zhang *et al*, 2000) or *Caenorhabditis elegans* (Long *et al*, 2002), an early stop in embryo development (Menand *et al*, 2002).

By using *Arabidopsis* T-DNA insertion lines in which the *AtTOR* gene is overexpressed and RNA interference (RNAi) lines

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with decreased levels of *AtTOR* expression, we show that *Arabidopsis* growth, seed yield, osmotic stress resistance, abscisic acid (ABA) and sugar sensitivity as well as polysome accumulation are positively correlated with levels of AtTOR messenger RNA.

RESULTS AND DISCUSSION

AtTOR expression level influences growth and seed yield

To investigate the role of the AtTOR kinase in post-embryonic growth and development, we first looked for hypomorphic (viable) *tor* alleles in the collections of T-DNA insertion mutants of *Arabidopsis*. *Arabidopsis* lines carrying T-DNA insertions upstream of the *AtTOR* translation start (as in the putative promoter or 5′ noncoding regions) segregated viable homozygous mutants (supplementary Fig S1 online). Insertion of the T-DNAs resulted in an increased steady-state level of the *AtTOR* mRNA either in the shoots and roots—mutants G548 and G166 from the Gabi collection (Fig 1A)—or only in the roots—mutants S7817 and S7846 from the Salk collection (Fig 1D). Both T-DNAs carry strong promoters at the border facing the 5′ leader sequence that could drive an increased transcription of the *AtTOR* gene. Such an alteration of transcriptional regulations following a T-DNA insertion just upstream of the translation start has already been described for the *Arabidopsis* WALL ASSOCIATED KINASE-LIKE 4 (*WAKL4*) gene (Hou *et al*, 2005). Thereafter, we tried to obtain *Arabidopsis* plants that underexpressed the *AtTOR* mRNA by introducing an *AtTOR*-derived hairpin construct under the control of the 35S promoter (constitutive RNAi; supplementary information online). Few transformants were obtained, but the 35-7, 65-1 and 36-6 lines showed a modest decrease in the accumulation of *AtTOR* mRNA (Fig 1A,D). These data indicate a strong counter-selection for the complete constitutive silencing of *AtTOR* and are consistent with *AtTOR* being an essential gene in plants.

Shoot and root growth was found to be enhanced in G166 and G548 *AtTOR*-overexpressing plants that were grown either in soil or in controlled hydroponic conditions (Fig 1B,C,E,F). In the S7846 and S7817 mutant lines in which overexpression of the *AtTOR* mRNA was restricted to the roots, only root growth was found to be enhanced (data not shown). Conversely, the partly silenced 35-7 and 65-1 lines showed reduced shoot and root growth (Fig 1). To gain a better understanding of the differences in leaf size, we measured the size of rosette leaves and adaxial epidermal cells (Fig 2; supplementary Fig S2 online). The area of rosette leaves increased gradually as the expression of *AtTOR* was augmented, and a comparable increase in epidermal cell size was observed. Interestingly, it has recently been shown that the epidermis determines shoot growth in *Arabidopsis* (Savaldi-Goldstein *et al*, 2007). Conversely, the elongation of etiolated hypocotyls of seedlings was not found to be affected by variations in *AtTOR* expression (supplementary Fig S3 online), which suggests that the *AtTOR* kinase does not control cell elongation, at least in etiolated hypocotyls. To determine the basis of the observed increase in root size for *AtTOR*-overexpressing lines, the structure of the root tip was examined by confocal microscopy (supplementary Fig S4 online). No marked changes in either the size of root meristem or organization were detected. Seed production was also higher in the plants overexpressing *AtTOR*

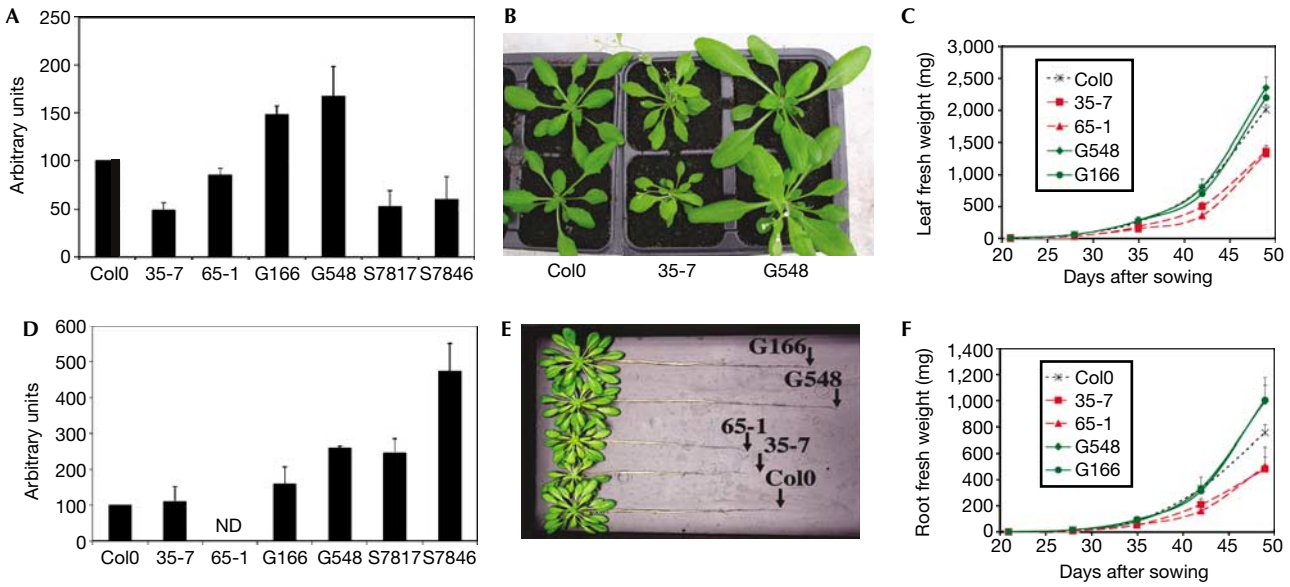

Fig 1 | The *AtTOR* gene regulates plant growth. (**A**,**D**) Expression of the *AtTOR* gene in the leaves (**A**) and roots (**D**) of the control line (Col0 wild type) and in RNAi-silenced (35-7 and 65-1) and RNAi-overexpressing lines (Gabi mutants G166 and G548; Salk mutants S7817 and S7846). Plants were grown *in vitro* for 15 days and relative expression levels were determined by real-time quantitative PCR (RT–PCR) with the control line as a reference (100 arbitrary units) in each experiment. The mean values ± s.d. of three independent experiments are shown. (**B**) Col0 WT plants, an RNAi-silenced line (35-7) and the G548 *AtTOR*-overexpressing line grown in the greenhouse for 36 days. (**C**,**F**) Leaf (**C**) and root growth (**F**) curves in hydroponic cultures of control Col0 (cross), G548 (diamonds), G166 (circles), 35-7 (squares) and 65-1 (triangles) lines. The mean values ± s.d. of three independent experiments are shown and correspond to the weight of individual plants. (**E**) Length of the root system in control, *AtTOR*-overexpressing and *AtTOR*-silenced lines. Plants were grown in hydroponic conditions for 49 days. ND, not determined; RNAi, RNA interference.

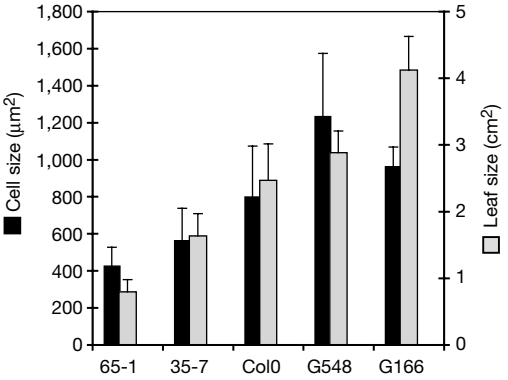

Fig 2 | *AtTOR* expression is correlated with the size of rosette leaves and epidermal cells. The size of rosette leaves and adaxial epidermal cells was determined for wild type (Col0), the *AtTOR*-silenced 65-1 and 35-7 lines, and for the G548- and G166-overexpressing lines. Leaf size (right axis) was measured on leaves 5 and 6 and averaged ($n = 4$). Epidermal cell size (left axis) was measured on the adaxial side of leaf 5 ($n = 3 \times 4$). See supplementary Fig S2 and supplementary information online for details.

in shoots, whereas it was reduced in *AtTOR*-silenced plants (Table 1). This difference was due to an increased or decreased growth and size of the inflorescence, and not due to changes in either seed or silique size (data not shown). It has already been reported that decreasing the level of the TOR kinase affects body size in *Drosophila* and *C. elegans* (Zhang *et al*, 2000; Long *et al*, 2002); however, this is the first evidence to our knowledge that elevated expression of the *TOR* gene can also result in an increased growth rate, fitness and size.

AtTOR controls resistance to osmotic stress

Nutrient availability is an important determinant of root growth (Malamy, 2005). To determine the role of *AtTOR* in linking the perception of nutritional information to root development, we studied the influence of the level of *AtTOR* expression on root size in response to nitrogen starvation or excess. Nitrogen starvation results in enhanced root growth, whereas an excess of nitrate inhibits root growth (Zhang *et al*, 1999) as a result of osmotic stress (Deak & Malamy, 2005). We observed that overexpression of *AtTOR* did not modify root length in N-limited conditions when compared with control plants (Fig 3A,B). Conversely, when plants were grown in the presence of excess nitrate, the *AtTOR*-overexpressing S7817 and S7846 lines showed a longer primary root (Fig 3A,B). This indicates that increased *AtTOR* expression relieves the inhibition of primary root growth by excess nitrogen.

To determine whether this effect was specific for nitrate or was linked to a more general osmotic effect, we used potassium chloride to change the osmotic potential of the growth medium. The primary root growth of the silenced lines 35-7 and 36-6 was reduced under high salt concentration, whereas the *AtTOR*-overexpressing lines G166 and G548 showed a longer primary root than the control line (Fig 3C). As for nitrate, the differences in primary root growth between these lines were much smaller when plants were grown in low-salt medium (data not shown). Seedlings of the 35-7- and 65-1-silenced lines also showed a higher sensitivity to osmotic stress after germination (Fig 3D). From these observations, we conclude that the level of *AtTOR* expression is inversely correlated with the sensitivity of the primary root length to salt concentrations and that a constitutive *AtTOR* expression might alleviate the effect of osmotic stress on root growth.

Table 1 | Production of seeds by *Arabidopsis* lines with increased (G548 and G166) or decreased (35-7 and 65-1) *TOR* expression

Genotype	Seed weight (mg/plant)	Length (µm)	Width (µm)
Col0	344.2 ± 76 (B)	479 ± 36	280 ± 20
35-7	185.1 ± 45.9 (A)	485 ± 32	282 ± 9
65-1	209.7 ± 55.5 (A)	ND	ND
G548	537 ± 159 (C)	477 ± 36	283 ± 24
G166	484 ± 65.9 (C)	ND	ND

Plants were grown to full maturity in greenhouse conditions. The weight of seeds harvested was measured for each plant. The mean values ± s.d. of seven independent plants are shown. The seed weight values that are statistically different (ANOVA followed by a Fisher test, $P < 0.05$) are labelled with different letters (A, B and C). The length and width of seeds are given in micrometers. ANOVA; analysis of variance; ND; not determined.

In *Schizosaccharomyces pombe*, a mutation in the *TOR* gene resulted in cells that are hypersensitive to osmotic stress (Weisman & Choder, 2001). In *Arabidopsis*, the expression of S6 kinases was found to be regulated by osmotic stress (Mizoguchi *et al*, 1995) and *Arabidopsis* plants overexpressing S6 kinase were also hypersensitive to osmotic stress (Mahfouz *et al*, 2006).

Silencing of *AtTOR* expression arrests plant growth

As levels of *AtTOR* mRNA were only partly reduced in constitutive RNAi lines, we investigated the effect of a more complete loss of *AtTOR* expression. We designed an ethanol-inducible system for the expression of double-stranded RNA based on the *alc* regulon of *Aspergillus* (Lo *et al*, 2005; supplementary information online). When silencing of the *AtTOR* gene was conditionally induced by ethanol at bolting, the amount of *AtTOR* mRNA was reduced to less than 20% of that of the control *alca:GUS* line (data not shown), and the growth of existing leaves was almost completely stopped when compared with control plants (Fig 4A). Ethanol-induced silencing of *AtTOR* expression triggered an early yellowing, which is linked to chlorophyll breakdown and is typical of leaf senescence (Fig 4A). Leaves from these plants also accumulated very high concentrations of soluble sugars (Fig 4C). Furthermore, we measured two and three times more glutamine synthetase and glutamate dehydrogenase activity, respectively, in leaves from silenced plants (data not shown). These variations in enzyme activities and levels of soluble sugar are usually associated with leaf senescence and nutrient remobilization in *Arabidopsis* (Diaz *et al*, 2005). This indicates that *AtTOR* activity is needed to restrain senescence and nutrient recycling.

When grown *in vitro* with increasing concentrations of ethanol, the silenced lines also showed an ethanol-dependent developmental arrest (Fig 4B,D). Silenced plantlets showed etiolated and unexpanded cotyledons, reduction and swelling of the hypocotyl and a lack of root development, as was more pronounced as ethanol concentration increased. This phenotype is reminiscent of *Arabidopsis* plantlets blocked by osmotic stress in a post-germinative checkpoint through ABA signalling or with an increased level of the ABA-insensitive 5 (ABI5) bZIP protein (Lopez-Molina *et al*, 2001). ABI5 is a crucial regulator of ABA

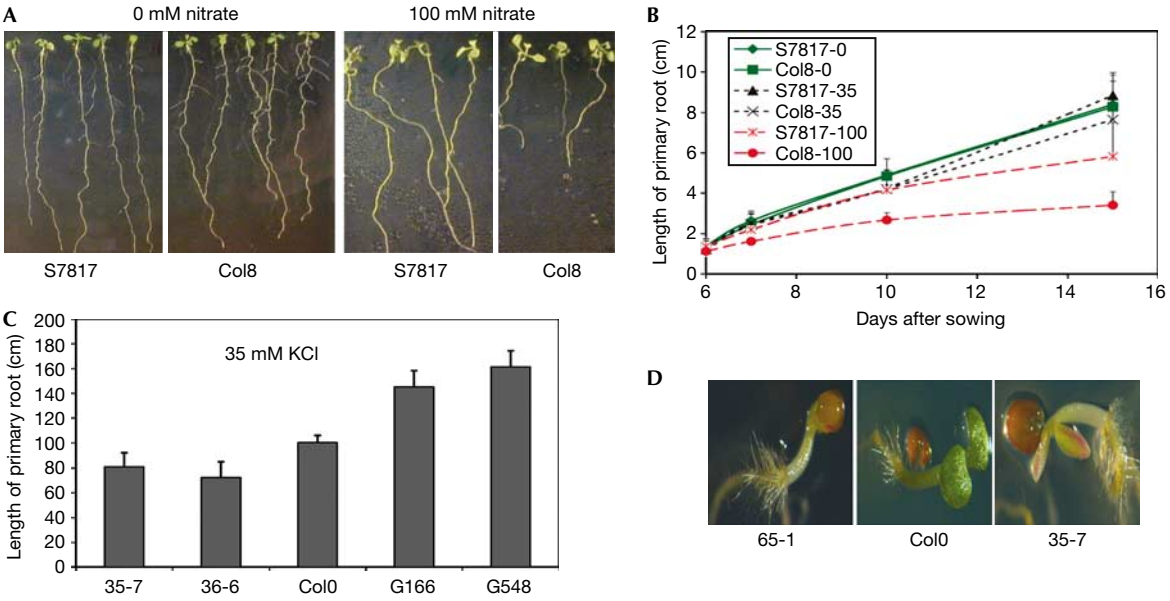

Fig 3 | *AtTOR* expression is correlated with sensitivity to osmotic stress. (**A**) Col8 wild type and S7817 seedlings were grown for 2 weeks on growth media without nitrogen (left) or on media supplemented with 100 mM nitrate (right). (**B**) Col8 wild type and *AtTOR*-overexpressing line S7817 were grown *in vitro* for 15 days with different nitrogen supplements (0, 35 or 100 mM nitrate). The length of the primary root was then measured at different time points. Values represent means ± s.d. of ten individual plants. The differences in root growth are only statistically significant for 100 mM nitrate. (**C**) Col0 wild type, *AtTOR*-silenced (35-71 and 36-6) and *AtTOR*-overexpressing (G166 and G548) lines were grown *in vitro* for 15 days with 35 mM KCl, and the length of the primary root was determined at the end of the culture. Values represent means ± s.d. of ten individual plants. (**D**) Seeds of Col0 wild type and of the *AtTor*-silenced 35-7 and 65-1 lines were sown on growth medium containing 5 mM mannitol. Representative 35-7 and 65-1 seedlings are shown.

signalling during post-germinative growth, and plantlets with silenced *AtTOR* also resembled *Arabidopsis* mutants of KEEP ON GOING, an E3 ubiquitin ligase required for ABI5 degradation (Stone *et al*, 2006). Furthermore, *AtTOR* RNAi lines were found to be hypersensitive to high sugar concentrations, whereas plants overexpressing *AtTOR* were less affected than the control plants (supplementary Fig S5 online). High sugar concentrations can retard seedling growth through the action of ABA (Finkelstein & Gibson, 2002) and we indeed observed a positive correlation between *AtTOR* expression and sensitivity to ABA (supplementary Fig S6 online). When grown on 2 μM ABA, about half of the seedlings from the silenced 35-7 line showed the same developmental arrest as those germinated on high concentrations of mannitol (Fig 3D) or when *AtTOR* expression was fully silenced (Fig 4D). Together, these results indicate that ABA or osmotic stress provokes a decrease in *AtTOR* expression or activity. The observation that the 35-7 RNAi line is more sensitive to osmotic stress than to ABA can be explained by the fact that osmotic stress signalling is mediated only in part by ABA (Finkelstein & Gibson, 2002). Thus, these results show that *AtTOR* expression is required for post-embryonic growth and might act as a relay for ABA signalling between environmental information and the post-germinative growth processes.

Silencing of *AtTOR* decreases accumulation of polysomes

Cell growth is intimately connected with ribosome biogenesis and protein translation (Wullschleger *et al*, 2006). We detected a marked decrease in the amount of soluble proteins in ethanol-silenced plants—6.5 mg and 8.3 mg of soluble proteins per g fresh weight of rosette for the RNAi6.1 and 6.2 lines, respectively, compared with 15.3 mg and 13.7 mg of soluble proteins per g fresh weight for the Landsberg *erecta* and *alca:GUS* control lines, respectively. The impact of *AtTOR* silencing on the efficiency of mRNA translation was then evaluated. On silencing, we observed a small but reproducible reduction in the abundance of high-molecular weight polysomes (Fig 5A). A decrease in polysome accumulation was also obtained after rapamycin treatment of yeast cells or of *Arabidopsis* plants expressing the yeast FKBP12 protein (Powers & Walter, 1999; Sormani *et al*, 2007). A marked increase in the 80S peak corresponding to the complete ribosome was observed after rapamycin treatment of yeast cells, but not after silencing of the *AtTOR* gene. Interestingly, changes in the expression level of the *Arabidopsis EBP1* (ErbB-3 epidermal growth factor receptor binding protein) gene, which shows a high sequence conservation to human EBP1—a nucleolar and cytoplasmic regulator of ribosome assembly and translation—affected organ growth in a manner similar to variations in *AtTOR* expression (Horvath *et al*, 2006). A higher expression of the *AtEBP1* gene was indeed detected in the *AtTOR*-overexpressing line S7817 when compared with Col0 after hybridization of CATMA arrays (urgv.evry.inra.fr/cgi-bin/projects/CATdb; D. Deprost & C. Meyer, unpublished data). These preliminary results were confirmed by quantitative real-time reverse transcriptase–PCR and the level of the EBP1 mRNA was found to be around twofold higher and lower in the *AtTOR*-overexpressing and *AtTOR*-silenced lines, respectively, when compared with the

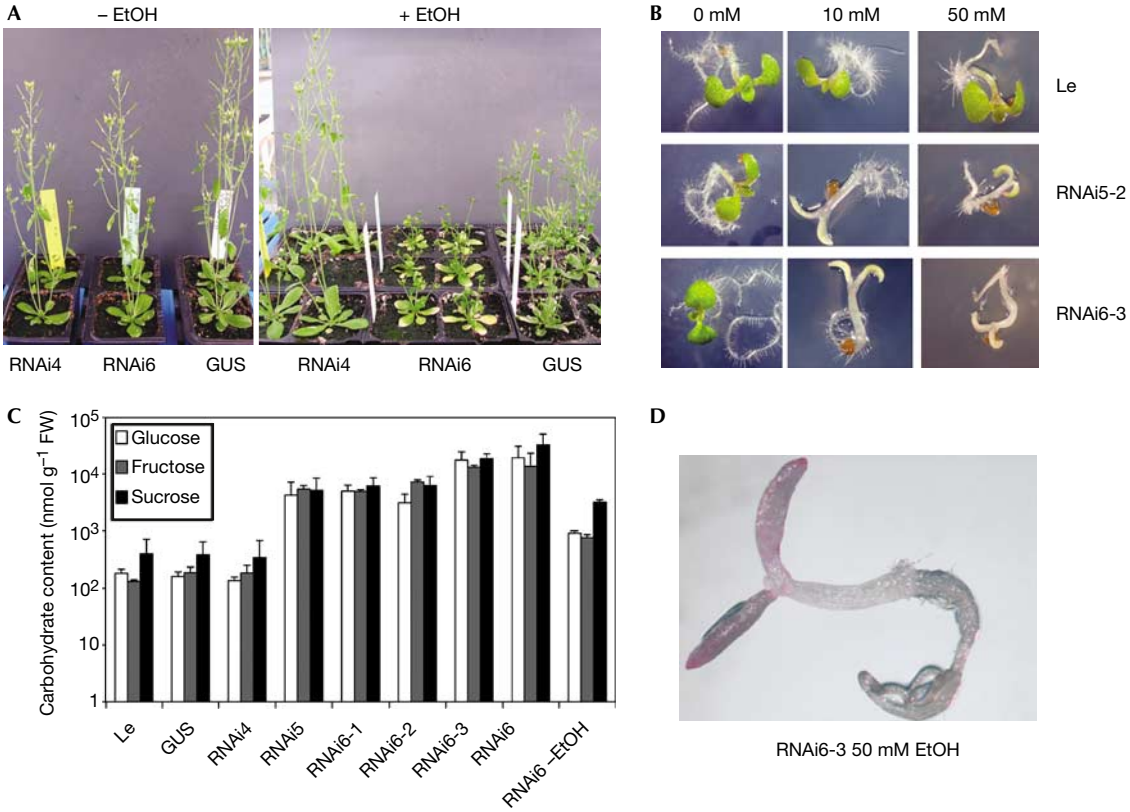

Fig 4 | Conditional silencing of *AtTOR* expression by ethanol-inducible RNA interference phenocopies in plants the action of rapamycin in animal and yeast cells. (**A**) After 6 weeks of culture without ethanol, *Arabidopsis* plants (Landsberg *erecta*) carrying either an *alcA:RNAi-TOR* construct (control line, RNAi4), a *35S:ALCR alcA:RNAi-TOR* construct (allowing for ethanol-inducible silencing of *AtTOR*, RNAi6) or a *35S:ALCR alcA:GUS* construct (GUS) were grown in the greenhouse for 10 days with or without 5% (v/v) ethanol (EtOH). (**B**) Landsberg *erecta* wild type (Le) and the progeny of two independent lines carrying a *35S:ALCR alcA:RNAi-TOR* construct were sown *in vitro* with (10 or 50 mM) or without ethanol. Representative seedlings are shown. (**C**) Comparison of carbohydrate contents (log scale) in the control (Le), GUS, RNAi4 and two independent *AtTOR*-silenced lines as well as in the homozygous progeny of the RNAi6 line grown with ethanol. The RNAi6 line was grown for 10 days with or without ethanol ($n = 8$), all other lines were grown for 27 days with ethanol ($n = 4$). The mean values ± s.d. are shown. (**D**) Close-up of an RNAi6-3 plantlet grown as in (**B**) with 50 mM ethanol. FW, fresh weight; RNAi, RNA interference.

level of EBP1 mRNA in control lines (Fig 5B). Furthermore, the expression of the *AtTOR* and *AtEBP1* genes was closely correlated when compared across various developmental stages (Genevestigator database; Zimmermann *et al*, 2004; supplementary Fig S7 online). Therefore, the EBP1 protein could be one of the targets of *AtTOR* in *Arabidopsis* and act downstream of the TOR kinase on the mRNA translation machinery.

In plants, the inactivation of *AtTOR* expression by inducible silencing phenocopies the action of rapamycin in animal and yeast cells—that is, an arrest of growth linked to a reduction of mRNA translation (Wullschleger *et al*, 2006). The use of conditional silencing of *AtTOR* expression is thus an efficient tool for decreasing TOR activity and will open new perspectives for the study of the TOR signalling pathway in plants.

Conclusion

Our data indicate that the TOR kinase could be important in the control of plant growth by exogenous information, partly through the regulation of mRNA translation and as a target of ABA signalling. Plant growth is an important yet poorly known biological process and only a few genes have been described that positively and negatively regulate the plant body size and the rate of growth (Horvath *et al*, 2006). The study of the *AtTOR* pathway is thus important for our understanding of the basic growth control mechanisms in plants.

METHODS

Constructs. For constitutive RNAi, a 300-bp fragment of the FKBP-rapamycin-binding (FRB) domain of *AtTOR* was amplified by PCR. The DNA fragment obtained was cloned in two opposite orientations in the pHANNIBAL plasmid under the control of the cauliflower mosaic virus 35S promoter. For the *alcA:RNAi-TOR* construct, a 190-bp fragment of the FRB domain of *AtTOR* was amplified by PCR and cloned in pHANNIBAL, in which the 35S promoter was replaced by the *alcA* promoter (O. Leleu, unpublished data). Induction of the ethanol switch was achieved by adding ethanol in the growth medium or by ethanol vapour from open Eppendorf tubes filled with 5% (v/v) ethanol in some

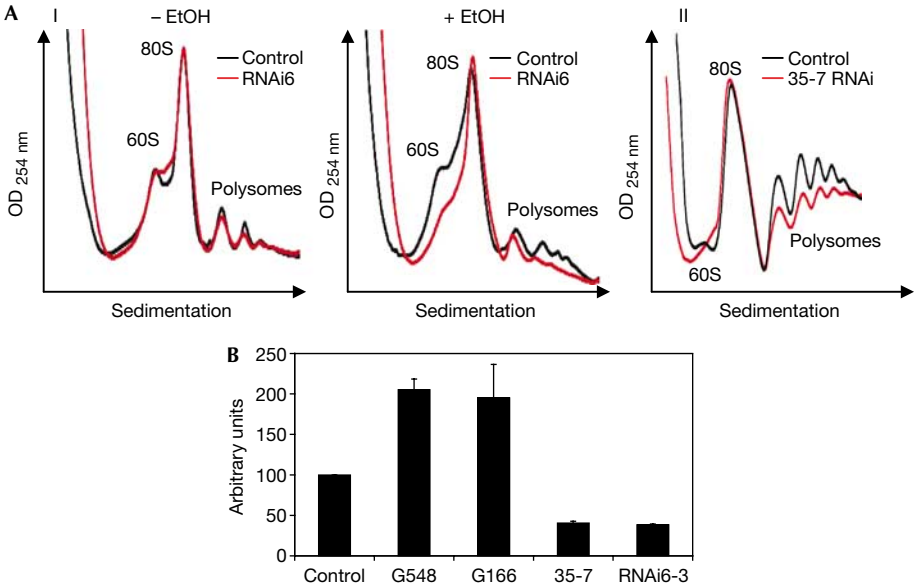

Fig 5 | Variations in *AtTOR* expression affect the messenger RNA translation machinery. (**A**) Absorbance profile at 254 nm of ribosomes purified by ultracentrifugation on a sucrose density gradient. The ribosomal pellet fraction was prepared (I) from shoots for the ethanol-inducible RNAi6 line, and (II) from plantlets grown *in vitro* in liquid medium for the partly silenced 35-7 line. Experiments were repeated at least three times and a typical experiment is shown for each line. Black lines correspond to control wild-type plants (Col0 and Landsberg *erecta* for, respectively, the 35-7 and RNAi6 lines). The positions of the 60S ribosomal subunits, monosomes (80S) and polysomes are indicated. (**B**) Increased and decreased expression of the *Arabidopsis EBP1* gene in, respectively, *AtTOR*-overexpressing (G548 and G166) and *AtTOR*-silenced (35-7 and ethanol-induced RNAi6-3) lines. RNA for relative quantification by real-time RT–PCR was obtained from leaves. The *EBP1* relative expression levels were determined as in Fig 1 with the control lines as reference (Col0 for G548, G166 and 35-7; ethanol-treated Landsberg *erecta* for RNAi6-3) in each experiment. The mean values ± s.d. of three independent experiments are shown. EBP, human ErbB-3 epidermal growth factor receptor binding protein; OD, optical density; RT–PCR, reverse transcriptase–PCR.

pots. The 35-7, 36-6 and 65-1 lines were obtained by transforming *Arabidopsis* (Col0 ecotype) plants with the construct described above for constitutive RNAi.

Plant material. The four T-DNA insertion mutants used in our study were derived from the Col8 (Salk collection, http://signal.salk.edu/) or the Col0 (Gabi collection, http://www.gabi-kat.de/) accession lines. Plants were grown in the greenhouse under standard conditions.

Polysome preparation. For the experiment using the 35-7 RNAi line, seeds (25 mg) were grown for 10 days in 20 ml of MS/2 medium with 1% sucrose at 25 °C under constant illumination and shaking. For the ethanol-inducible RNAi lines, control and ethanol-treated shoots were collected without flower buds. See supplementary information online for details.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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